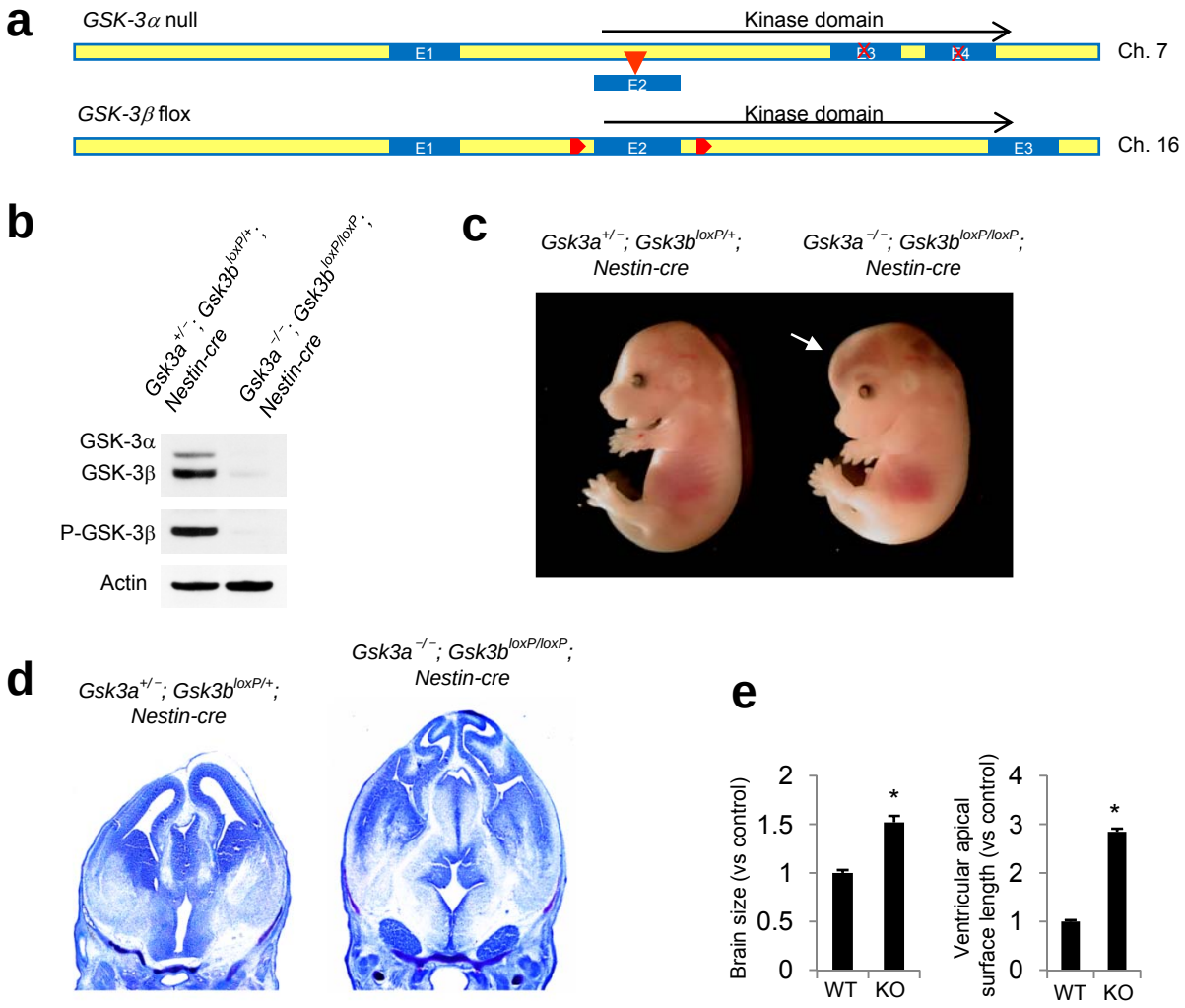
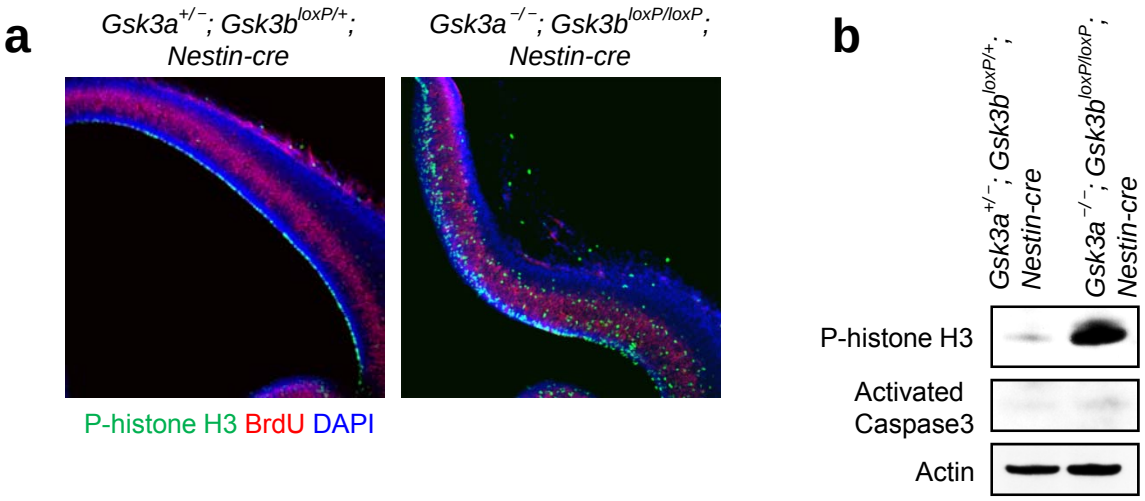
GSK-3 is a master regulator of neural progenitor homeostasis

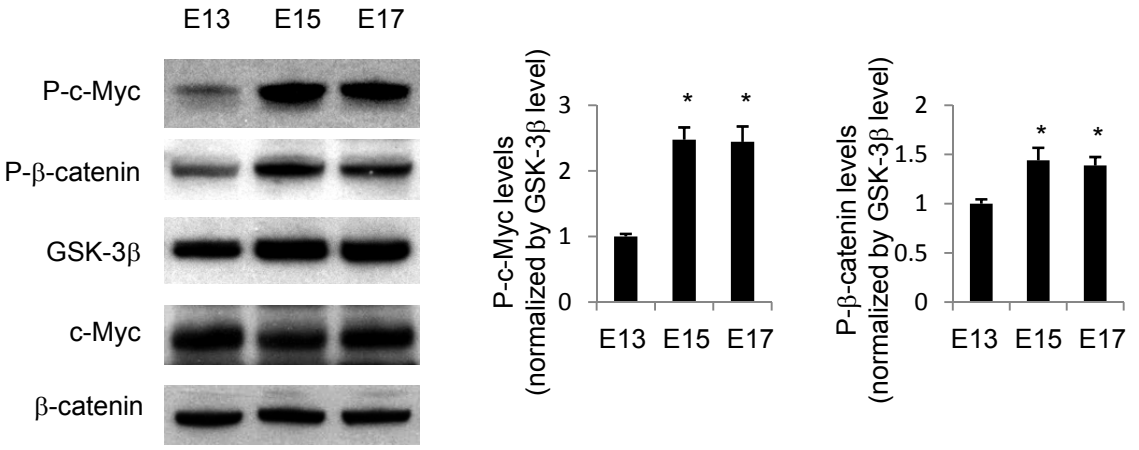
Woo- Yang Kim[1], Xinshuo Wang[1], Yaohong Wu[1], Bradley Doble[3], Satish Patel[2], James Woodgett[2], and William Snider[1]*



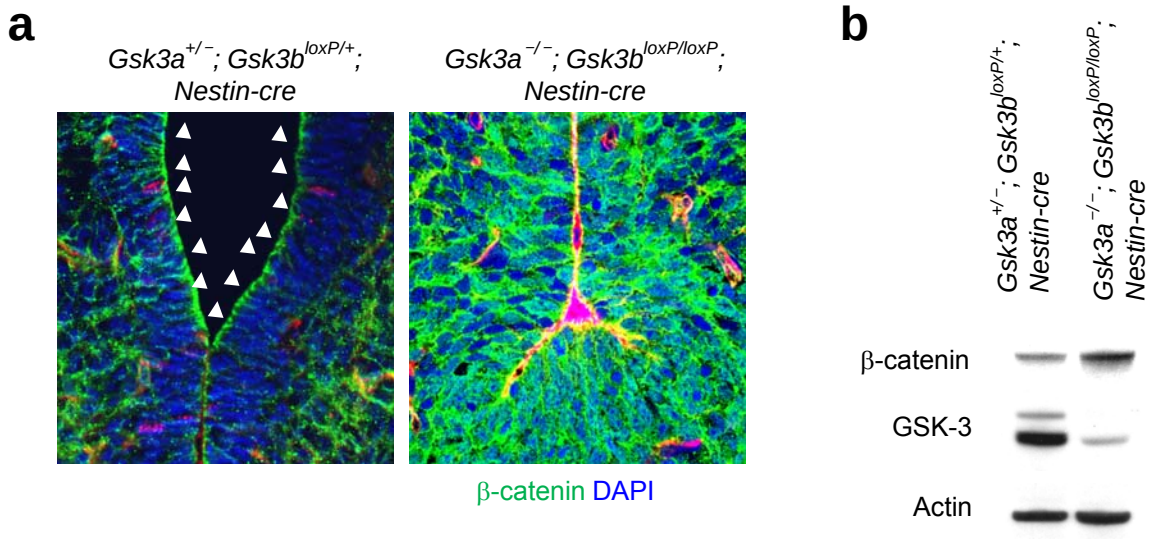
Supplementary Fig. 1 GSK-3 deletion results in brain enlargement and infolding of the cortical wall. (**a**) Simple diagrams of *Gsk3a* and *Gsk3b* knockout alleles. Schematics show the first few exons of *Gsk3a* and *Gsk3b* genes and the targeting strategies . For the *Gsk3a* knockout, exon2 was disrupted to generate a reading frame shift leading to a premature stop codon. For *Gsk3b*, exon 2 was flanked by loxP sites. (**b**) Western blot shows GSK-3β protein is almost completely eliminated in brains of *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* mice at E13.5. GSK-3a protein is absent as expected. (**c**) *GSK-3a*$^{-/-}$ *GSK-3b*$^{F/F}$*Nestin-cre* embryos had noticeably larger heads by E15.5 (arrow) compared to control mice (*Gsk3a*$^{+/-}$*; Gsk3b*$^{loxP/+}$*; Nestin-cre*). (**d**) Coronal sections at E13.5 with cresyl violet staining shows marked convolutions and infolding of the developing cortical wall in *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* mice. The cortical wall is smooth in controls at this age. Ventral brain areas including the ganglionic eminence and ventral thalamus are also enlarged in the mutant. (**e**) Quantification of brain size and ventricular apical surface length. Data shown are mean ±SEM (n=3 animals each condition). * indicates significant difference when compared with controls at $p<0.01$.



Supplementary Fig. 2 Increased progenitor proliferation in *Gsk3a* $^{-/-}$*; Gsk3b* $^{loxP/loxP}$*; Nestin-cre* brain. (**a**) Low magnification pictures of phospho-histone H3 and BrdU stained dividing cells in cerebral cortex sections. *Gsk3a* $^{-/-}$*; Gsk3b* $^{loxP/loxP}$*; Nestin-cre* cortex sections showed many more dividing cells positive for phospho-histone H3 and BrdU compared to control sections. (**b**) Western blots showed that the level of phospho-histone H3 was markedly increased in the *Gsk3a* $^{-/-}$*; Gsk3b* $^{loxP/loxP}$*; Nestin-cre* brain tissues. There was no change in the level of activated caspase 3.

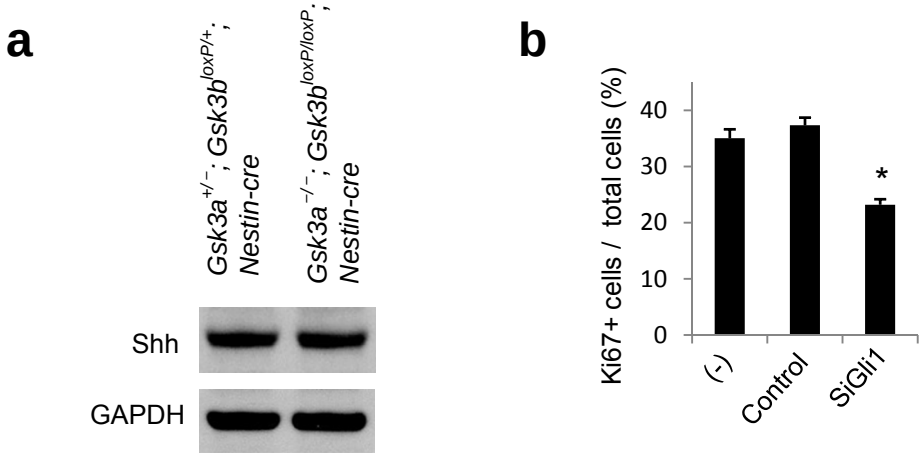


Supplementary Fig. 3 GSK-3 activity increases during brain development. *Left panel:* Western blotting showed that GSK-3 activity is developmentally regulated. Levels of phospho-c-Myc and phospho-β-catenin in brain lysates were measured at 3 developmental stages E13, E15, and E17. The phosphorylation levels increased significantly between E13 and E15. *Middle and right panels:* Quantification of Western blotting. Levels of phospho-β-catenin and phospho-c-Myc were normalized to GSK-3β levels.

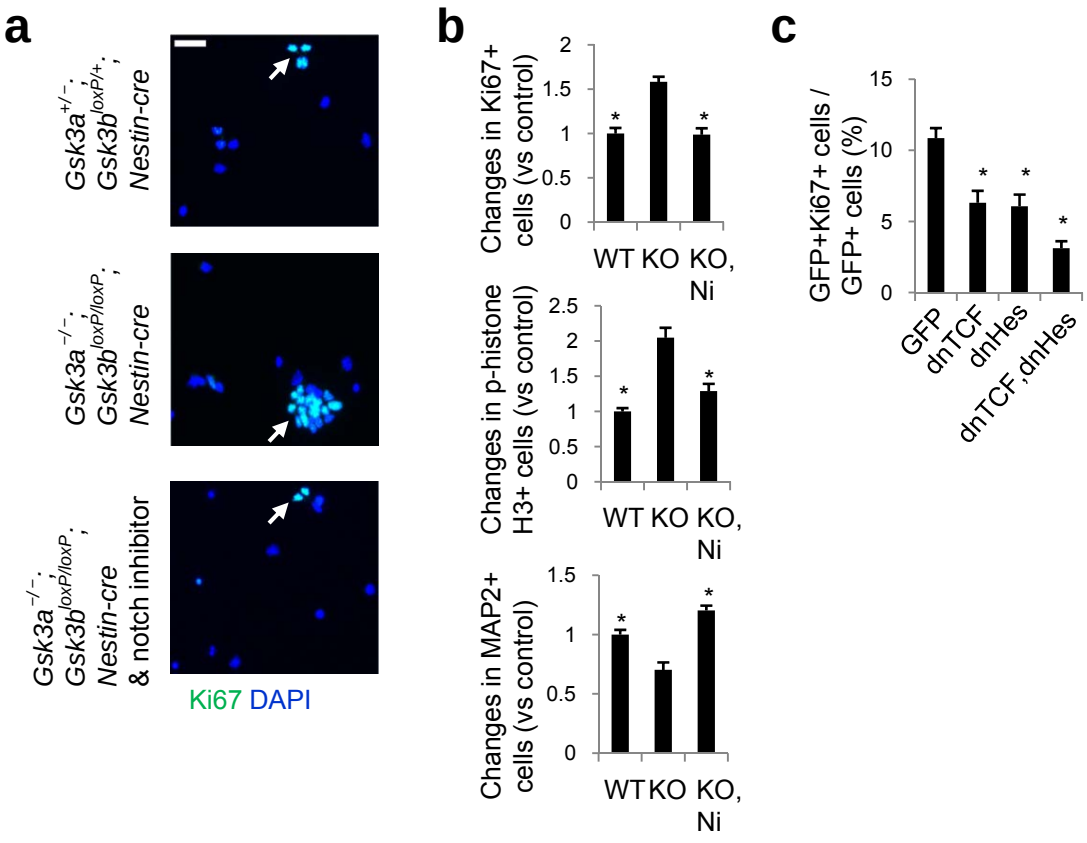


Supplementary Fig. 4 β–catenin is upregulated and inappropriately distributed in *Gsk3a* $^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* spinal cord. (**a**) Loss of the spatial expression pattern of β–catenin in *Gsk3a* $^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* spinal cord. β-catenin was enriched in apical surfaces along central canal in control. However, most cells of *Gsk3a* $^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* spinal cord highly expressed β-catenin. (**b**) Western blotting showed that GSK-3 proteins were eliminated in *Gsk3a* $^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* spinal cord and that cellular β-catenin level is markedly upregulated.

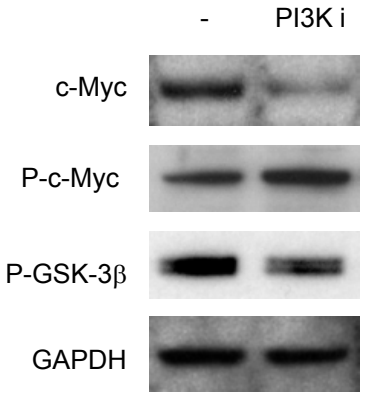
Nature Neuroscience: doi:10.1038/nn.2408



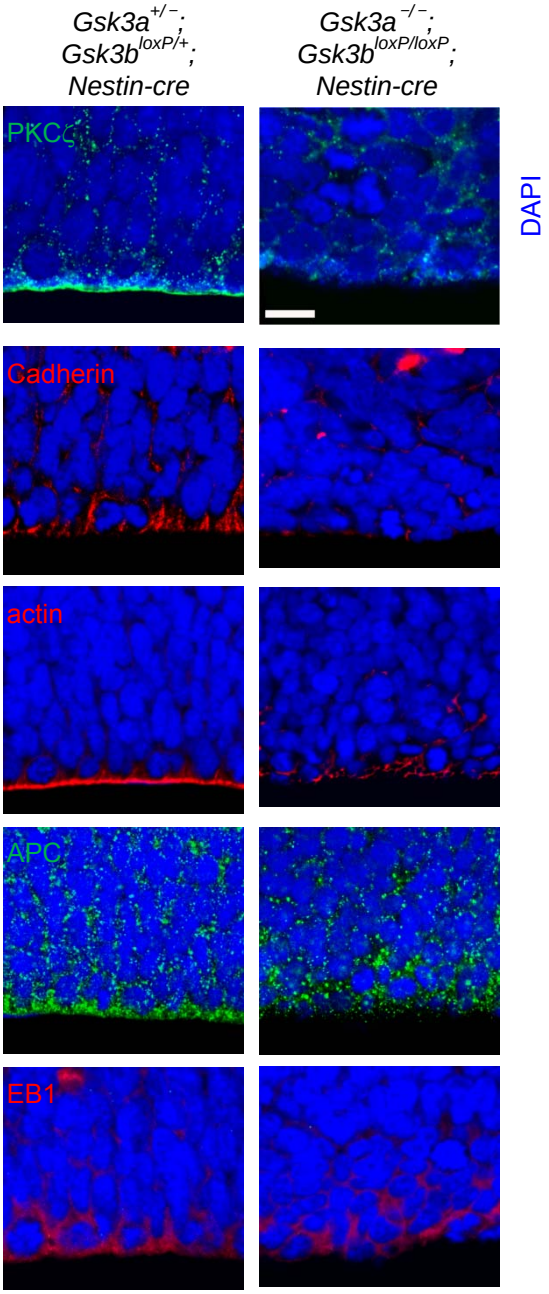
Supplementary Fig. 5 Activated Shh signaling is associated with hyperproliferation of GSK-3 mutant neural progenitors. (**a**) Western blot showed that levels of Shh protein was not changed in GSK-3 mutant brain lysates. (**b**) Elimination of Gli1 decreased proliferation. Dissociated *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* progenitors were transfected with either control (random) or Gli1 siRNA and then cultured for 48 hours, followed by immunostaining with anti-Ki67 antibody. Percentages of Ki67-positive cells per total cells were counted for quantification. *p<0.01 (vs control).



Supplementary Fig. 6 Inhibition of Notch signaling suppresses proliferation of neural progenitors from *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* cortex. (**a**) Progenitor hyperproliferation in *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* brain is mediated by Notch signaling. Analysis of Ki67 and phospho-histone H3 staining showed that more cells divided in cultures from *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* cortex. Treatment with a gamma secretase inhibitor DAPT reduced the abnormal proliferation and increased the number of MAP2-positive neurons in *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* cortical cells in culture. Scale bar: 20 mm. (**b**) Quantification of Ki67, phospho-histone H3, and MAP2 positive cells in the *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* cortical cultures treated with a gamma secretase inhibitor. *p<0.01 (vs. KO). (**c**) Inhibition of either β-catenin signaling or Notch signaling partially blocks proliferation of control progenitor cells (*Gsk3a*$^{+/-}$*; Gsk3b*$^{loxP/+}$*; Nestin-cre*) in culture. The percentages of Ki67- and GFP- positive cells to total transfected cells (GFP-positive) were counted for quantification.



Supplementary Fig. 7 Inhibition of PI3K decreases c-Myc level in cortical progenitors. Cortical cells from E14 brains were cultured and treated with LY294002. c-Myc levels were decreased in the presence of LY294002. Changes in phospho-c-Myc (T58) and phospho-GSK-3b (S9) levels indicated the efficiency of PI3K inhibition.



Supplementary Fig. 8 GSK-3 regulates apical-basal polarity in developing cerebral cortex. Staining for cell polarity and cell adhesion markers showed loss of cell polarity and disruption of apical-junctional complexes in *Gsk3a*$^{-/-}$*; Gsk3b*$^{loxP/loxP}$*; Nestin-cre* brain sections. Scale bar: 10 mm.

Nature Neuroscience: doi:10.1038/nn.2408